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Characterization of Sucrose-Negative *Pasteurella multocida* Variants, Including Isolates from Large-Cat Bite Wounds

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To validate the identification of *Pasteurella multocida*-like bacteria negative for acid formation from sucrose, including isolates from bite wounds caused by large cats, 17 strains were phenotypically and genotypically characterized. Phylogenetic analysis of partially sequenced *rpoB* and *infB* genes showed the monophyly of the strains characterized and the reference strains of *P. multocida*. The sucrose-negative strains formed two groups, one related to reference strains of *P. multocida* and the other related to a separate species-like group (taxon 45 of Bisgaard). DNA-DNA hybridization further documented the species-like nature of this group. Ribotyping showed the heterogeneity of all strains except four strains that shared the same ribotype and that were isolated from bovine lungs. Phylogenetic analysis by 16S rRNA sequence comparison showed the monophyly of the strains characterized and the reference strains of *P. multocida*. Two strains isolated from leopard bite wounds were related to the type strain of *P. dagmatis*; however, they represented a new taxon (taxon 46 of Bisgaard), in accordance with their distinct phenotypic and genotypic identifications. The present study documents that sucrose-negative strains of *P. multocida*-like bacteria belong to two genotypically distinct groups. The study further confirms the phenotypic heterogeneity of *P. multocida* strains and documents two new species-like taxa of *Pasteurella* related to *P. multocida*. Until diagnostic tools have been further elaborated, special care should be taken in the identification of *Pasteurella*-like bacteria isolated from bite wounds caused by large cats. The evidence of phenotypic and genotypic divergence calls for the further development of PCR tests and DNA sequencing to document doubtful isolates.

Pasteurella multocida has been isolated from a multitude of hosts (26), and different lineages of *P. multocida* may be responsible for various diseases in both birds and mammals (9, 15). Human infections with *P. multocida* are in most cases of animal origin and are most often related to the bites of carnivores. However, other types of infections are also occasionally reported (25, 28, 34).

Significant variations in the phenotypic properties of *P. multocida* have been reported (27), leading to confusion in the definition and identification of this organism. Mutters et al. (37) reclassified the genus *Pasteurella* on the basis of DNA-DNA hybridization studies. Three clusters of *P. multocida* showing 84 to 100, 91 to 100, and 89 to 100% DNA reassociation between strains subsequently described as *P. multocida* subsp. *multocida*, *P. multocida* subsp. *gallicida*, and *P. multocida* subsp. *septica*, respectively, were identified. Representatives of the existing capsular types were found to be closely related on the basis of DNA-DNA hybridization (44), despite the diversity of disease manifestations and hosts. This is contradictory to the diversity shown by outer membrane protein profiling (17, 18, 19), multilocus enzyme electrophoresis (5), and ribotyping (43). The study of Petersen et al. (43) showed a great diversity of ribotypes among strains classified as *P. multocida* subsp. *multocida*, *P. multocida* subsp. *gallicida*, and *P. multocida* subsp. *septica*. However, comparisons of the sequences of the 16S rRNA gene and the *atpD* gene (which encodes the β subunit of ATP synthase) confirmed the overall homogeneity of *P. multocida* (43). The study of Kuhnert et al. (33) also showed that variant phenotypes of *P. multocida* shared at least 98.5% 16S rRNA sequence similarity with the recognized subspecies of this species.

P. avium and *P. canis* were reported as new species by Mutters et al. (36, 37). Both species were separated into two biovars. Strains of *P. canis* biovar 2 and *P. avium* biovar 2 and strains of *P. multocida* deviating in key phenotypic characters were subsequently genotyped to examine their relationship with *P. multocida* (13). Surprisingly, these investigations allowed the reclassification of *P. multocida* to include biovars 2 of both *P. avium* and *P. canis*, and on the basis of this background, the existence of biovars 2 of *P. avium* and *P. canis* was questioned. The redefined species *P. multocida* is genotypically homogeneous, although phenotypically diverse lineages exist with respect to the key characteristics ornithine decarboxylase, indole, and mannitol fermentation, which have been regarded as essential for identification of *P. multocida* to the species level (13).

P. multocida and *P. multocida*-like bacteria have occasionally been isolated from the wounds of humans bitten by large cats, like lions and tigers (6, 25, 29, 48, 50). Similar organisms have also been isolated from the dental-gingival junction of several species of large cats (50). The majority of these isolates do not ferment sucrose (25, 50) and, consequently, differ from *Pasteurella* sensu stricto, which is defined as sucrose positive (9). Strain SSI P 876, proposed as "*P. leonis*" (Table 1), was isolated from a man bitten by a lion, and this strain was found to

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TABLE 1. Strains used for investigation of *P. multocida*, including isolates from large cats and sucrose-negative variants

Species and identification	Strain[a]	Country, yr of isolation	Source (animal, disease, or organ)	Ribotype	PCR result	Capsular type	GenBank accession no.		
							16S rRNA	*rpoB*	*infB*
Reference strains									
P. multocida subsp. *multocida*	NCTC 10322^T (ATCC 43137^T)			5	+[b]	A	AF294410[c]	AY170216[c]	AJ289690[c]
P. multocida subsp. *gallicida*	NCTC 10204^T (ATCC 51689^T)			6	+	A	AF326323[c]	AY362969[c]	AY683511
P. multocida subsp. *septica*	CIP A125^T (NCTC 11995^T, ATCC 51687^T)			7	+	A	AF326325[c]	AY362970[c]	AY683512
P. canis	NCTC 11621^T					NT[d]	AY362919[c]	AY314038[c]	
P. multocida subsp. *septica*	5[e]	Germany	Calf pneumonia	ND[f]	+	A	AY316314[c]	AY683493	AY683513
P. multocida subsp. *septica*	25[e]	Germany	Calf pneumonia	ND	+	A	Identical to AY316314[c]	AY683494	AY683514
P. multocida subsp. *septica*	A 285/86[e]	Germany, 1986	Calf pneumonia	ND	+	A	Identical to AY316314[c]	AY683495	AY683515
P. multocida subsp. *septica*	W 208[e]	Germany	Bovine organs	ND	+	A	AY316317[c]	AY683496	AY683516
Strains investigated									
P. multocida subsp. *multocida*, ornithine, indole, and sucrose negative	B80/20[g]	United Kingdom	Bovine pneumonia	4	+	A	Identical to AY316316[c]	AY683497	AY683517
P. multocida subsp. *multocida*, ornithine, indole, and sucrose negative	B80/26[g]	United Kingdom	Bovine pneumonia	4	+	A	Identical to AY316316[c]	AY683498	AY683518
P. multocida subsp. *multocida*, sucrose negative	W 819	United Kingdom	Bovine pneumonia	4	+	A	AY683485	AY683499	AY683519
P. multocida subsp. *multocida*, mannitol and sucrose negative	RA 12/2[e]	United Kingdom	Bovine pneumonia	10	+	A	AY316316[c]	AY683500	AY683520
P. multocida subsp. *septica*, mannitol, indole, and sucrose negative	K 323[e]	Denmark	Bovine pneumonia	1	+	A	Identical to AY316316[c]	AY683501	AY683521
P. multocida subsp. *septica*, indole and sucrose negative	Schmid W 87-227-9 (HIM 1057-3)	Germany, 1987	Sheep	3	+	A	Identical to AY316317[c]	AY683502	AY683522
P. multocida subsp. *septica*, sucrose and mannitol negative	Younan S_2 (HIM 996-8, MCCM 00657)	Syria, 1986	Sheep, nose	2	+	A	Identical to AF224298[c]	AY683503	AY683523
P. multocida subsp. *septica*, Ornithine, sucrose and mannitol negative	288[g]	Germany	Calf pneumonia	4	+	A	Identical to AY316315[c]	AY683504	AY683524
Bisgaard taxon 45	14589/75[g]	United Kingdom	Source unknown	11	+	A	AY683486	AY683505	AY683525
Bisgaard taxon 45	CDC F 4484	United States	Man, tiger bite wound	9	+	NT	AY683487	AY683506	AY683526
Bisgaard taxon 45	CDC G 9955	United States	Man, tiger bite wound	ND	+	ND	AY683488	ND	ND
Bisgaard taxon 45 "*P. multocida* subsp. *tigris*"	ATCC BAA-600	United States	Man, tiger bite	12	+	NT	AY057994[c]	AY683507	AY683527
Bisgaard taxon 45 "*P. leonis*"	SSI P 876 (HIM 969-4, FsK11447, MCCM 00659)	Kenya, 1984	Man, bite from lion	8	−	NT	AY683489	AY683508	AY683528
Bisgaard taxon 45	47182	United Kingdom, 1975	Man, lion bite wound	ND	ND	ND	ND	ND	ND
Bisgaard taxon 45	HIM 1004-6 (*Pasteurella* sp. strain Schmid 351)	Germany, 1967	Chipmunk	13	+	A	AY683490	AY683509	AY683529
Bisgaard taxon 46	CDC A996	United States	Man, leopard bite wound	14	−	NT	AY683491	AY683510	AY683530
Bisgaard taxon 46	CDC F 4646	United States	Man, leopard bite wound	ND	−	ND	AY683492	ND	ND

[a] ATCC, American Type Culture Collection; NCTC, National Collection of Type Cultures; CIP, Collection de I'Institut Pasteur; HIM, Hygienischer Institut Marburg; MCCM, Microbiology Culture Collection Marburg; CDC, Centers for Disease Control and Prevention; SSI, Statens Serum Institut.
[b] +, positive reaction by the *P. multocida*-specific PCR of Miflin and Blackall (35).
[c] Accession number of a sequence determined in a previous report.
[d] NT, no type obtained by the capsular PCR test.
[e] From an earlier investigation by Christensen et al. (13).
[f] ND, not determined.
[g] From an earlier investigation by Bisgaard et al. (4).

be genotypically related to *P. multocida* (30); however, the polyamine pattern of the strain differed from that of the type strain (7). Strain ATCC BAA 600 (Table 1) was isolated from a girl bitten by a tiger. The name "*Pasteurella multocida* subsp. *tigris*" was proposed for this strain on the basis of phenotypic characterization and 16S rRNA gene sequence comparisons (8). The phenotypic characteristics reported were in accordance with those of *P. multocida*, except for the lack of fermentation of sucrose and mannitol, the fermentation of which being which specific characteristics of *P. multocida* (39). Disregarding the results for sucrose and mannitol fermentation, the combination of sorbitol fermentation and a lack of dulcitol, xylose, and trehalose fermentation would have identified the isolate as *P. multocida* subsp. *multocida* (39).

Among 1,268 cultures of *P. multocida* characterized by Heddleston (27), none were reported to be sucrose negative. Among some 1,500 strains characterized in the laboratory of one of the authors (M. Bisgaard, unpublished data), only 17 were sucrose negative.

The objective of the present study was to characterize sucrose-negative variant strains of *P. multocida*-like bacteria, including isolates obtained from large cats, in order to investigate their taxonomic position and host relationship and to identify methods for the identification of these bacteria.

MATERIALS AND METHODS

Selection of bacterial strains and phenotypic characterization. A total of 25 bacterial strains were investigated, including reference strains and strains that have been described previously (4, 8, 13) (Table 1). Phenotypic characterization of 15 strains was performed on the basis of 79 characteristics, as reported previously (4).

Ribotyping. Ribotyping of 17 strains selected to represent phenotypic variants, including type strains of the three subspecies of *P. multocida*, was done as described by Christensen et al. (16). Briefly, 6 μg of DNA was digested with the HpaII enzyme (New England Biolabs, Beverly, Mass.) at 37°C for 2 h. The digests were separated on a 0.8% agarose gel for 16 h at 35 V. The gel was stained in ethidium bromide, and the DNA was vacuum blotted onto a nitrocellulose filter. The filter was reacted with a 16S-23S rRNA-specific probe overnight at 56°C. Ribotype patterns were rendered visible with a digoxigenin wash and block buffer kit (Roche A/S, Hvidovre, Denmark), according to the instructions of the manufacturer. Bacteriophage λ DNA digested with HindIII (New England Biolabs) was included as a size marker. Ribotype patterns were analyzed with Gelcompar software (version 4.0; Applied Maths, Kortrijk, Belgium), and Dice coefficients were used to construct a neighbor-joining dendrogram with the PHYLIP software package (24).

Sequencing of 16S rRNA, *rpoB*, and *infB* genes. The 16S rRNA gene sequences of 18 strains were determined, and the *rpoB* and *infB* sequences of 21 strains were compared (Table 1). The bacteria were cultured overnight in brain heart infusion broth (Difco, Detroit, Mich.) at 37°C. The fragments amplified by PCR were purified and cycle sequenced on an automatic sequencer (ABI 377; Chemistry Guide; Applied Biosystems, Foster City, Calif.), as described recently (14, 31, 40). DNA sequencing resulted in at least 1,278, 470, and 446 bp of the 16S rRNA, *rpoB*, and *infB* genes, respectively.

Analysis of sequence data. Searches for homologous DNA sequences in the GenBank database were performed with the BLAST program (1). Pairwise similarities were calculated with the BESTFIT program (Wisconsin Sequence Analysis Package; Genetics Computer Group, Madison, Wis.). Phylogenetic analysis by parsimony analysis was performed with the PROTPARS and CONSENSE programs with the default settings included with the PHYLIP package (24). Maximum-likelihood analysis, including bootstrap analysis, was performed with fastDNAml software (41) run on a Linux (version 7.2)-compatible server.

DNA-DNA hybridization. DNA-DNA hybridization was determined for 11 pairs of strains by the microwell method (11) with the modifications reported previously (13), including growth with EDDA (ethylenediamine-*N*,*N*′-diacetic acid) and hyaluronidase to limit capsule formation of isolates with capsular type A.

***P. multocida*-specific PCR and capsular typing by PCR.** The PCR procedure of Miflin and Blackall (35) was followed, as reported recently (13). Briefly, a loopful of an overnight culture was taken from the surface of a blood agar plate and suspended in sterile water. The suspension was boiled, the cells were spun down, and the supernatant was used as the template for the PCR. PCR was performed with primers PM23F1 and PM23R2 (35). After electrophoresis and ethidium bromide staining, an amplicon of 1,432 bp could be visualized under UV light. Capsular typing by PCR was performed as described by Townsend et al. (49).

ITS analysis. PCR typing by the 16S-23S rRNA internal transcribed spacer (ITS) analysis approach was performed for five strains, as reported by Christensen et al. (13).

Nucleotide sequence accession numbers. The nucleotide sequences described in this report have been deposited in GenBank under accession numbers AY683485 to AY683530 and are indicated in Table 1 and Fig. 2 to 4.

RESULTS

Phenotypic characterization. Seventeen of the 79 characteristics investigated varied between the 22 strains analyzed (Table 2), all of which were in accordance with the species description of *P. multocida* described by Mutters et al. (39) and Christensen and Bisgaard (9) (Table 2). Eight and four strains were classified as *P. multocida* subsp. *septica* and *P. multocida* subsp. *multocida*, respectively, while nine strains remained unclassified. Seven of these were phenotypically related to *P. multocida* subsp. *gallicida* but could be separated from this subspecies by sucrose and glycerol utilization, and some could be separated by the formation of gas from (+)-D-glucose (Table 2). These organisms, tentatively named Bisgaard taxon 45, were obtained from tiger ($n = 3$) and lion ($n = 2$) bite wounds in humans, a chipmunk, and an unknown source. Two strains, both of which were obtained from leopard bite wounds in humans, also remained unclassified. Both of these isolates differed from the type strain of *P. dagmatis* in (−)-D-sorbitol, sucrose, and trehalose fermentation, key characteristics for the separation of members of the family *Pasteurellaceae*. For the same reasons mentioned above, these organisms might represent a new taxon and are consequently named Bisgaard taxon 46.

Ribotyping. Seventeen strains were compared by ribotyping, including the three type strains of *P. multocida*. Between three and nine bands were registered at 13 positions in 14 ribotypes (Fig. 1). One band of approximately 5 kb was common to all strains. One group included the two type strains of *P. multocida* subsp. *multocida* and *P. multocida* subsp. *gallicida* and seven other strains, all of which were sucrose negative. These nine strains were closely related and shared at least four bands. Four strains even shared the same ribotype. The remaining strains were less uniform with respect to ribotype. This group included taxa 45 and 46; however, strain RA 12/2 of *P. multocida* subsp. *multocida* was also located with these strains by ribotyping.

16S rRNA sequence comparison. Identical 16S rRNA gene sequences were found between sucrose-negative strains HIM 996-8 and 288 and sucrose-positive strain 214 (GenBank accession no. AF224298) (13). Strains B 80/20, B 80/26, and K 323 had the same sequence as previously sequenced strain RA12/2 (GenBank accession no. AY316316) (13), and strain HIM 1057-3 showed a sequence identical to the previously published sequence of sucrose-positive strain W208 (GenBank accession no. AY316317) (13). This shows that sucrose-posi-

TABLE 2. Variable phenotypic characteristics of *P. multocida* and taxon 45 of Bisgaard

Characteristic	Result for[a]:					
	P. multocida subsp. *multocida* NCTC10322^T	*P. multocida* subsp. *gallicida* NCTC10204^T	*P. multocida* subsp. *septica* NCTC11995^T	*P. multocida* subsp. *septica* (eight atypical strains[b])	*P. multocida* subsp. *multocida* (four sucrose-negative strains)	Taxon 45 of Bisgaard (seven strains)
Oxidase	+	+	+	d (4)[c]	d (1)	+
Ornithine decarboxylase	+	+	+	d (4)	d (2)	+
Indole	+	+	+	d (2)	d (2)	+
Glycerol	−	−	+	−	−	d (6)
(+)-L-Arabinose	−	+	−	−	−	−
(−)-D-Arabinose	−	−	(+)	−	−	−
(+)-D-Xylose	(+)	−	+	d (3)	−	d (5)
Dulcitol	**−**[d]	**+**	**−**	**−**	**−**	**d** (3)
(−)-D-Mannitol	(+)	+	+	d (2)	d (2)	**−**
(−)-D-Sorbitol	**(+)**	**+**	**−**	**−**	**+**/(+)	**+**
(−)-L-Fucose	−	−	(+)	−	**d** (1)	−
Gas from (+)-D-glucose	−	−	−	−	−	**d** (3)
Lactose	−	−	−	d (1)	−	−
ONPG[e]	−	−	−	d (1)	−	−
Sucrose	+	+	+	d (4)	−	**−**
Trehalose	+	−	+	+	+	**−**
PNPG[f]	+	−	+	+	+	**d** (1)

[a] +, positive within 1 to 2 days; (+), positive within 3 to 14 days; −, negative after 14 days; d, variable.
[b] Including four reference strains (Table 1).
[c] The number of positive strains is given in parentheses.
[d] Key characteristics used for separation of taxa are in boldface.
[e] ONPG, β-galactosidase test performed with *o*-nitro-phenyl-D-galactopyranoside.
[f] PNPG, α-glucosidase test determined with 4-nitrophenyl-α-D-glucopyranoside.

tive and -negative isolates can have identical 16S rRNA sequences.

The phylogenetic analysis based on 16S rRNA gene sequence comparison showed the monophyly of all strains of *P. multocida*, including the sucrose-negative variants of both *P. multocida* and taxon 45 (Fig. 2, left panel). The level of 16S rRNA gene sequence similarity was 98.4% or higher within this group, and the level of similarity was at least 98.6% between all the strains and the type strain of *P. multocida*. If only the type strain of *P. multocida* subsp. *septica* was included in the phylogenetic analysis, a tendency for two subgroups to appear within the major *P. multocida* group was observed (Fig. 2, left panel). Strains classified as taxon 45 (strains ATCC BAA 600, 14589/75, SSI P 876, HIM 1004-6, and CDC F 4484) and the type strain of *P. multocida* subsp. *septica* showed at least 99.4% gene sequence similarity. At least 98.4% similarity was found between this group and the other group, including the type strains of the other two subspecies. Low bootstrap values were found for this tree, including the node supporting the two groups of *P. multocida*-like strains. The reason for these low bootstrap values was probably the low levels of sequence variation. For this reason the position of *P. multocida* subsp. *septica* should also be taken with caution. Repeat analysis after the inclusion of 11 additional *P. multocida* subsp. *septica* sequences published previously by Kuhnert et al. (33) changed the positions of the type strain and related strains such that they formed a new group with strain CDC A 996 and CDC F 4646 (taxon 46) and the type strain of *P. dagmatis* (Fig. 2, right panel). In this new version of the tree, the remaining strains of *P. multocida* could no longer be separated from taxon 45.

The two strains of taxon 46 formed a monophyletic group with the type strain of *P. dagmatis* if only the type strain of *P. multocida* subsp. *septica* was included (Fig. 2, left panel). The highest degree of similarity found for strain CDC A 996 was to strain F 4646 (99.6%) and the type strain of *P. dagmatis* (98.4%). At most, 97.4% similarity to the sucrose-negative and -positive strains of *P. multocida* investigated was found.

The reason for the instability of the phylogenetic trees based on 16S rRNA sequences when a few more related strains are included is probably caused by a low degree of sequence variation. For this reason, sequencing of the housekeeping genes *infB* and *rpoB* was initiated.

***infB and rpoB* sequence comparison.** The two groups of sucrose-negative strains found by ribotyping (except for RA 12/2) were also recognized by phylogenetic analysis of partially sequenced *infB* and *rpoB* genes (Fig. 3 and 4). The four strains of *P. multocida* isolated from wounds caused by large-cat bites and the strain isolated from a chipmunk formed their own monophyletic group. This group was provisionally named taxon 45 of Bisgaard by phenotypic classification (see above). Strains classified as Bisgaard taxon 46 and represented by strain CDC A 996 were place into a group unrelated to *P. multocida* and taxon 45, and comparison of the *rpoB* and *infB* sequences did not allow classification of Bisgaard taxon 46 with *P. dagmatis* (Fig. 3 and 4).

DNA-DNA hybridization. On basis of the present results and results published by Eckert et al. (22), Stenzel (47), and Christensen et al. (13), an overall high degree of DNA reassociation of at least 78% seems to exist for strains classified in the single subspecies of *P. multocida* (Table 3), in accordance with the

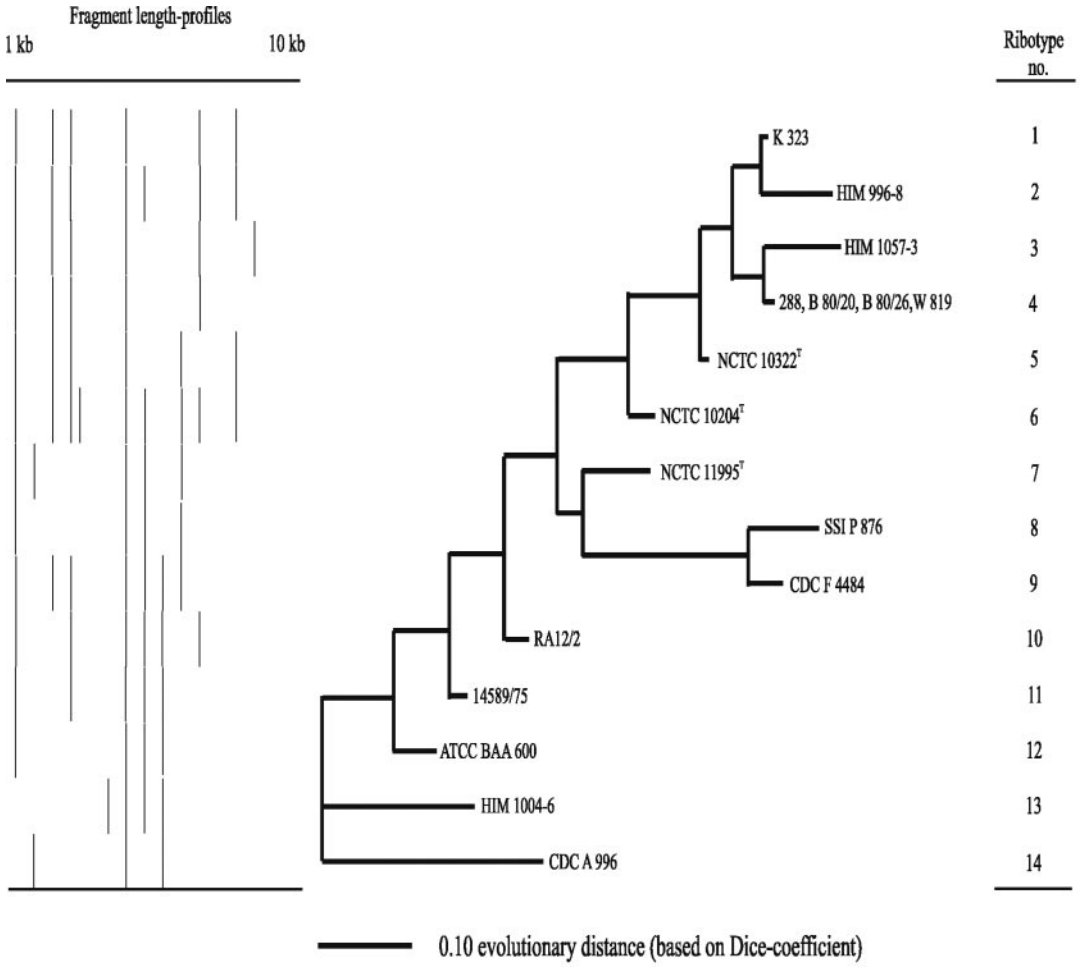

FIG. 1. Genotypic relationships between *P. multocida* and related atypical strains of *Pasteurella* investigated by ribotyping. Strains with identical profiles for specific ribotypes are indicated at the branch tips. The dendrogram was based on neighbor-joining cluster analysis of the Dice coefficients formed by comparison of the fragment profiles.

general levels of DNA reassociation of the three subspecies (37). Slightly lower DNA reassociation values were observed between subspecies of *P. multocida*, which represent phenotypically deviating strains. For these strains the rates of DNA reassociation varied between 53 and 91% (Table 3). Strains classified as taxon 45 all showed DNA binding values below the species level (31 to 51%), with the strains representing the three subspecies of *P. multocida*; however, 90% DNA reassociation was found between the two strains of taxon 45 (Table 3). When the *rpoB* and *infB* sequence analysis results are compared with the ribotyping results, taxon 45 seems to differ from *P. multocida* and probably represents a new species-like taxon of *Pasteurella*.

PCR. With the exception of a single strain, all strains of taxon 45 surprisingly tested positive by the PCR test of Miflin and Blackall (35) for *P. multocida*. Both strains of taxon 46 tested negative by the PCR test. In addition, the results of capsular typing remained negative for this taxon; however, negative capsular typing results were also observed for strains of taxon 45. The typeable strains were all of type A.

ITS analysis. ITS analysis showed that strains 288, B 80/20, and B 80/26 had the same profile as the type strain of *P. multocida*; however, another profile was found for strains 14589/75 and HIM 1004-6 (data not shown). These results confirm the existence of two groups of sucrose-negative strains that are recognized by ribotyping, *rpoB* and *infB* sequence comparison, and DNA-DNA hybridization.

DISCUSSION

The present study has documented that sucrose-negative strains of *P. multocida*-like bacteria belong to two genotypically distinct groups. One group that is genotypically similar to *P. multocida* contained strains mainly isolated from cows with pneumonia, whereas the other group was isolated mainly from large-cat bite wounds and formed a new species-like taxon (taxon 45 of Bisgaard). The study supplements previous investigations of phenotypically divergent *P. multocida* strains (5, 13, 23, 42), including V factor-dependent strains of *P. multocida* subsp. *multocida* isolated from pigs with pneumonia (32). The documentation of phenotypic variations in key characteristics of *P. multocida*, including maltose (42), ornithine decarboxylase, indole, and mannitol (13), and sucrose (the present study)

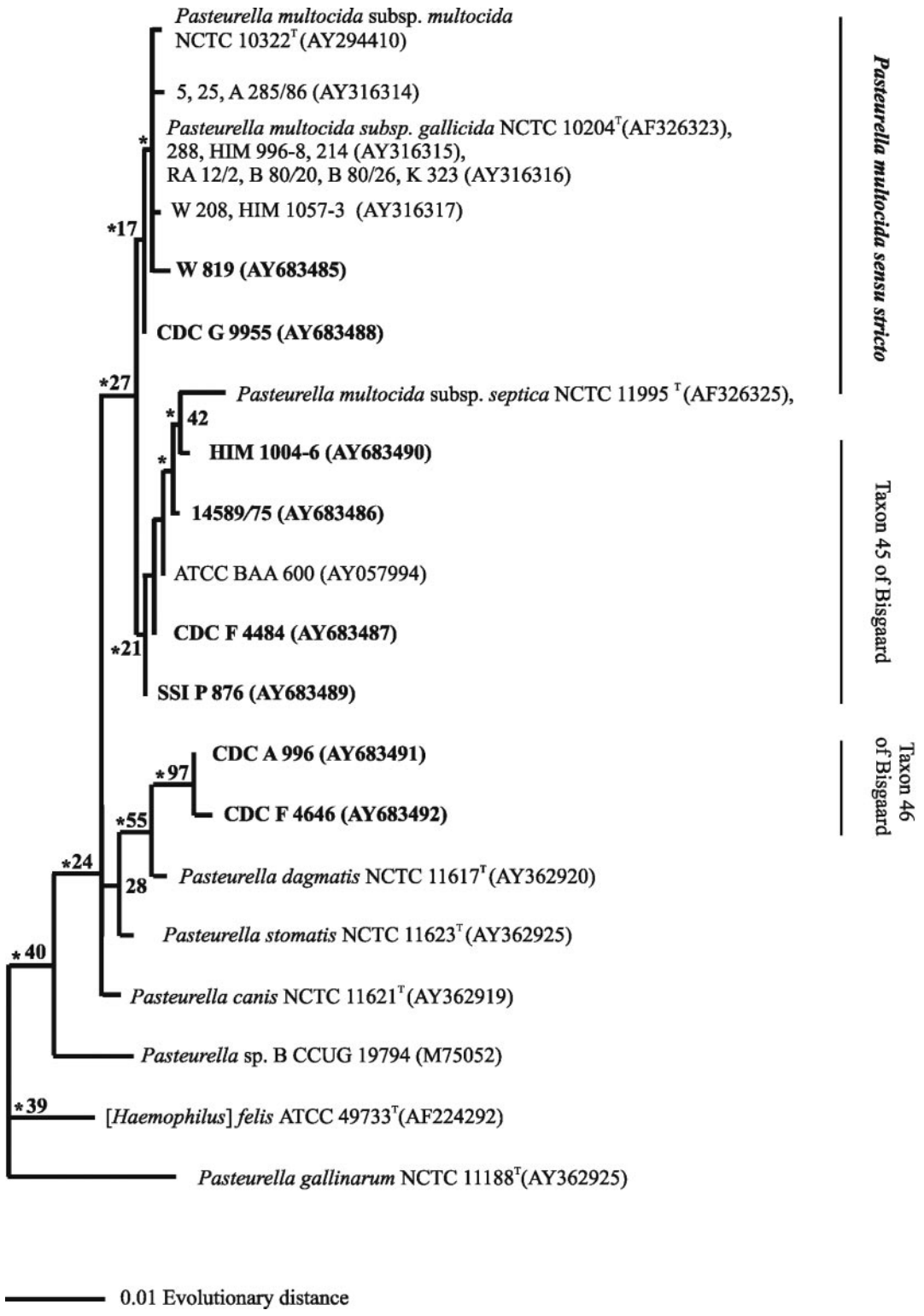

FIG. 2. Phylogenetic relationships between strains of *Pasteurella*, including the type strain of *P. multocida* subsp. *septica* only (left panel) and 11 additional strains of this taxon (right panel), based on maximum-likelihood analysis of 16S rRNA gene sequences. The support for monophyletic groups by bootstrap analysis is indicated as percentages. Nodes recognized by maximum-parsimony analysis are marked with an asterisk. Strains sequenced in the present study are in boldface type.

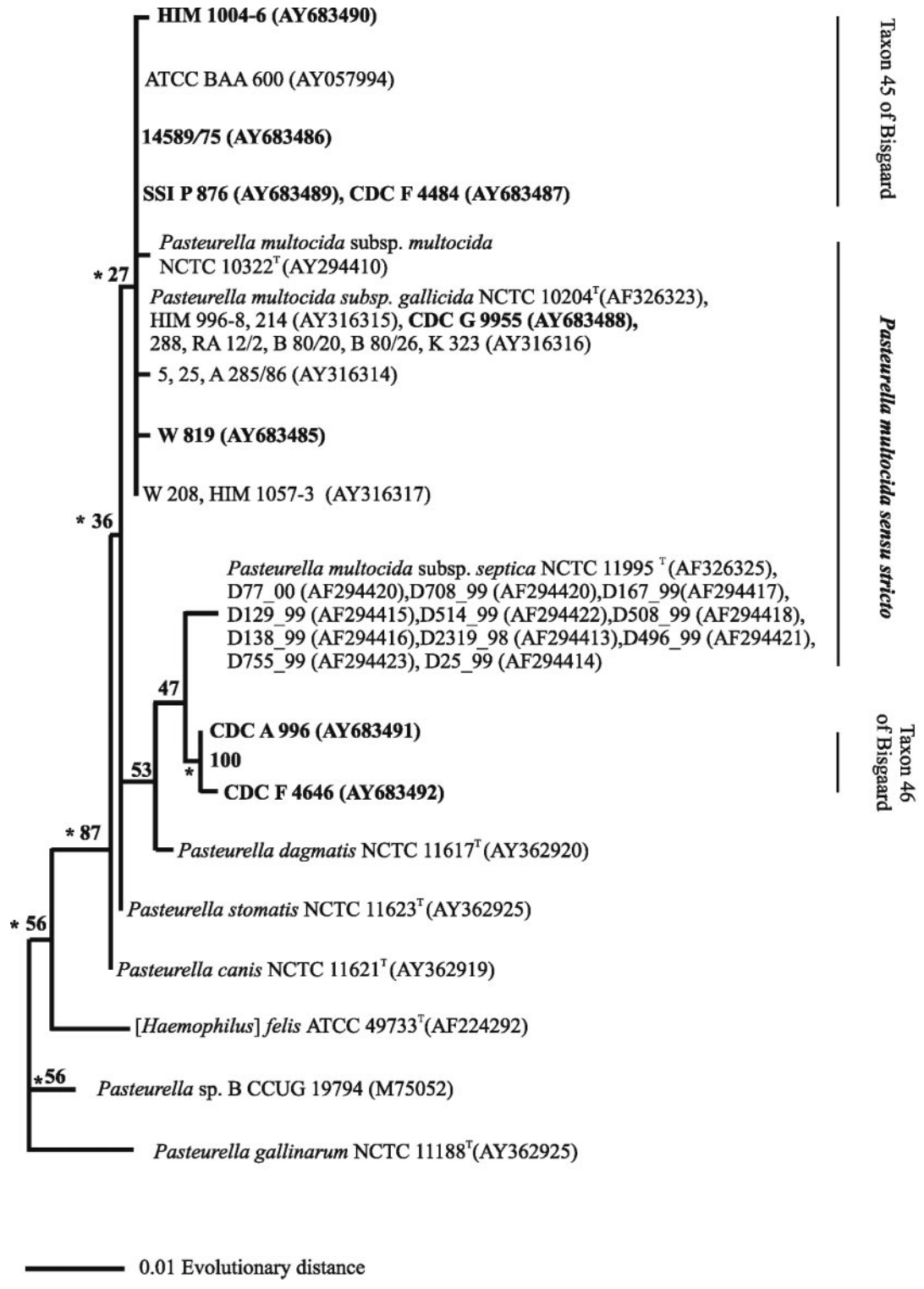

FIG. 2—*Continued*.

fermentation, makes routine diagnosis on the basis of phenotypic characteristics difficult and uncertain.

The present investigation confirms previous difficulties associated with the use of 16S rRNA gene sequence comparisons for separation of the subspecies of *P. multocida*, as well as with the use of phenotypic characteristics for the separation of *P. gallinarum* and related species (12, 43). This problem has also been reported for other taxa (46). On the basis of 16S rRNA gene sequence comparisons, the four strains of *P. multocida* subsp. *septica* were expected to show a tight relationship with

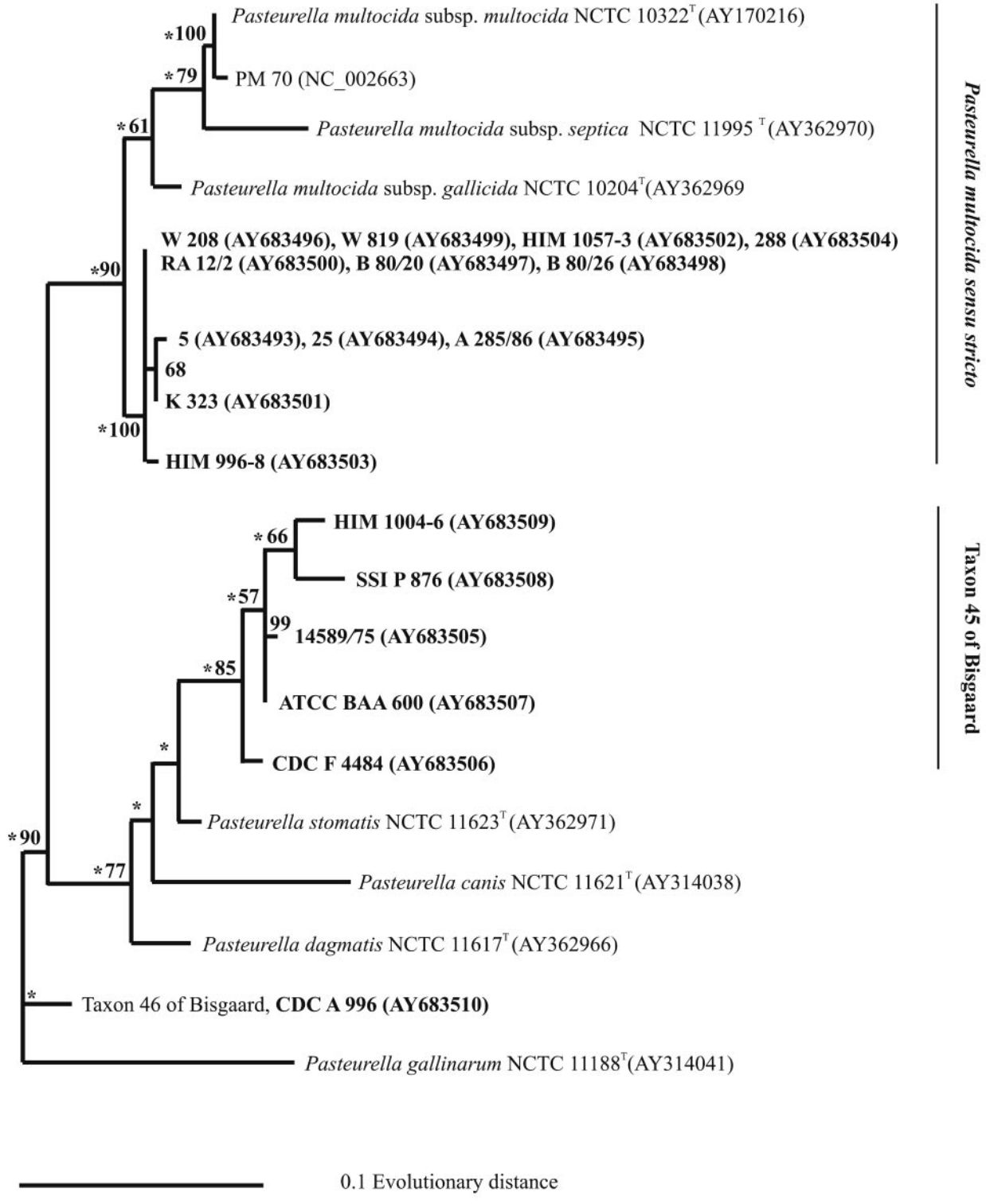

FIG. 3. Phylogenetic relationships between strains of *Pasteurella* based on maximum-likelihood analysis of the *rpoB* gene DNA sequences. The support for monophyletic groups by bootstrap analysis is indicated as percentages. Nodes recognized by maximum-parsimony analysis are labeled with an asterisk. Strains sequenced in the present study are in boldface type.

the type strain of this taxon. Surprisingly, these strains were more closely related to *P. multocida* subsp. *multocida* (Fig. 2). The four strains probably represent sorbitol-negative variants of *P. multocida* subsp. *multocida*, and they will therefore be misidentified as *P. multocida* subsp. *septica* by phenotyping. Similar observations were made in a previous study (13).

The 16S rRNA phylogenetic tree that incorporated all strains of *P. multocida* and related taxa was rather unstable in

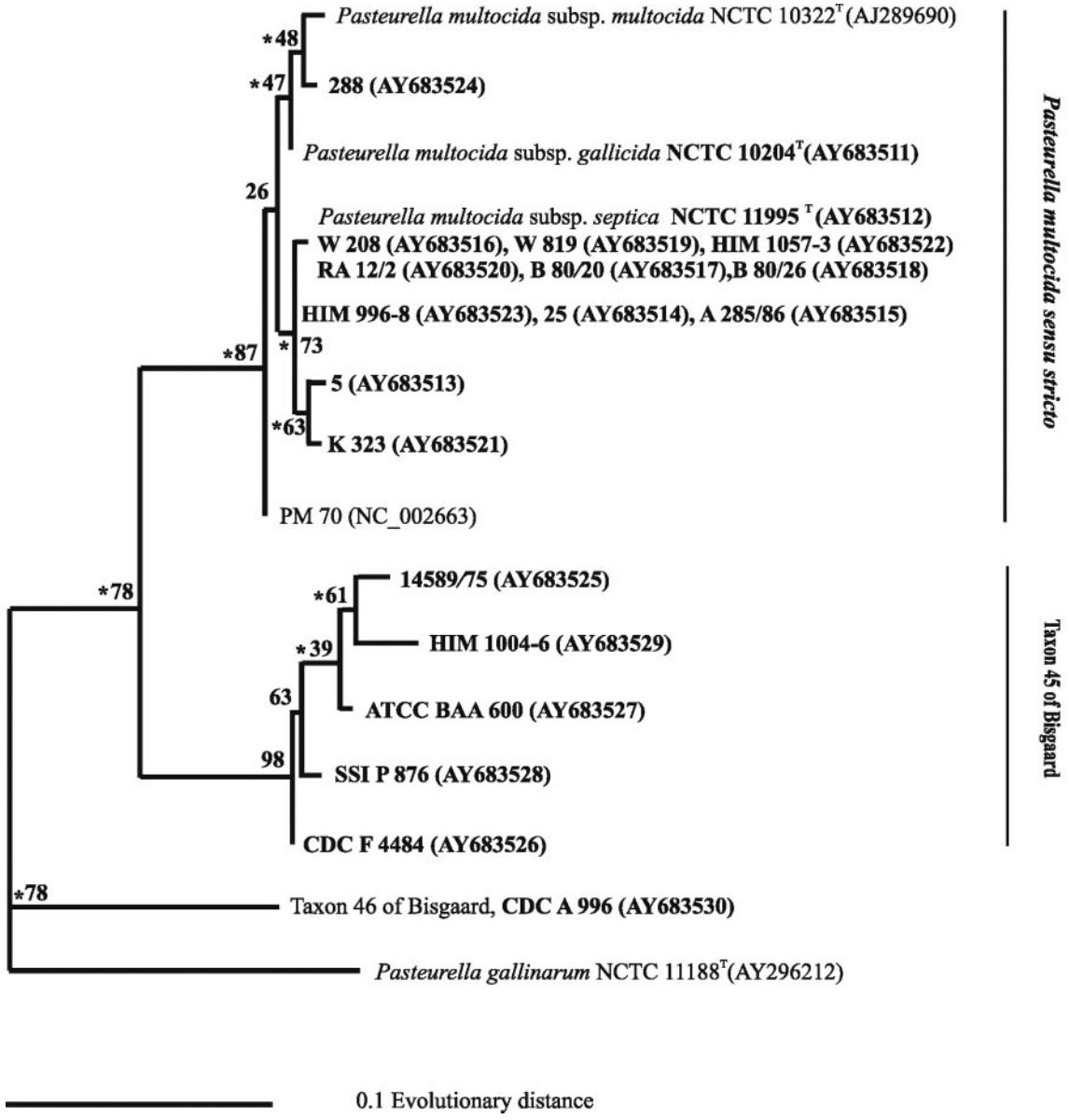

FIG. 4. Phylogenetic relationships between strains of *Pasteurella* based on maximum-likelihood analysis of the *infB* gene DNA sequences. The support for monophyletic groups by bootstrap analysis is indicated as percentages. Nodes recognized in maximum parsimony analysis are labeled with an asterisk. Strains sequenced in the present study are in bold face type.

relation to perturbations of input sequences (Fig. 2). For this reason, housekeeping gene sequences were compared. The present study is the first to use housekeeping gene sequence comparison for classification of a larger set of strains of *P. multocida* and related bacteria. Davies et al. (20) recently used housekeeping gene sequence comparison for the diagnostic comparison of *P. multocida* isolates. The results for the two genes *rpoB* and *infB* analyzed in the present study corresponded well, and the analysis seemed to provide a deeper resolution at the species level compared to that achieved by 16S rRNA gene sequence comparison. Recently, *infB* sequence comparison has successfully been used for phylogenetic analysis of *Haemophilus* and *Actinobacillus* sensu stricto and *rpoB* sequence comparison has been used for investigation of *Histophilus somni* and members of the family *Pasteurellaceae* (3, 14, 31, 40). Although comparison of housekeeping gene sequences has been recommended for classification to the species level (46), a consensus has not been reached.

The results of phenotypic and phylogenetic analyses of sequences of the housekeeping genes *rpoB* and *infB* point to the existence of a new sucrose-negative species of *Pasteurella* isolated mainly from the wounds of bites caused by large cats and tentatively named taxon 45 of Bisgaard. The resolution obtained by 16S rRNA sequence analysis did not enable a clear separation of the group from *P. multocida*. However, except for one strain, the members of taxon 45 were recognized by ribotyping. Two strains from the divergent group showed a DNA reassociation rate at the species level of 90% and were well

TABLE 3. DNA-DNA hybridization between strains of *P. multocida* and taxon 45 of Bisgaard in relation to that for sucrose-negative variants

Taxon	Strain(s)	Taxon	Strain(s)	% DNA reassociation
P. multocida subsp. *multocida*	NCTC 10322^T	*P. multocida* subsp. *multocida*	B 80/20	78 (9)[a]
P. multocida subsp. *septica*	5	*P. multocida* subsp. *septica*	A 285/86	96 (9)[b]
	HIM 996-8		HIM1057-3	95 (7)[c]
	W 208		A 285/86	94 (19)[b]
	25		A 285/86	81 (13)[b]
P. multocida subsp. *multocida*	RA 12/2	*P. multocida* subsp. *septica*	A 285/86	91 (18)[b]
	NCTC 10322^T		HIM 996-8	66 (7)[c]
	NCTC 10322^T		A 285/86	63 (17)[b]
P. multocida subsp. *multocida*	B 80/20	*P. multocida* subsp. *gallicida*	NCTC10204^T	70 (9)
P. multocida subsp. *gallicida*	NCTC 10204^T	*P. multocida* subsp. *septica*	A 285/86	53 (12)[b]
P. multocida subsp. *multocida*	NCTC10322^T	Taxon 45 of Bisgaard	HIM 1004-6	48 (12)
	NCTC10322^T		SSI P 876	41 (4)[c]
	NCTC10322^T		14589/75	40 (4)
	RA 12/2		HIM 1004-6	34 (8)
P. multocida subsp. *gallicida*	NCTC10204^T	Taxon 45 of Bisgaard	14589/75	42 (8)
	NCTC10204^T		HIM 1004-6	36 (15)
P. multocida subsp. *septica*	HIM 1057-3	Taxon 45 of Bisgaard	SSI P 876	51 (3)[c]
	HIM 1057-3		HIM 1004-6	50 (2)[c]
	HIM 996-8		HIM 1004-6	47 (6)[c]
	5		HIM 1004-6	43 (6)
	NCTC 11995^T		SSI P 876	40 (8)[c]
	HIM 996-8		SSI P 876	38 (5)[c]
	25		HIM 1004-6	38 (4)
	A 285/86		HIM 1004-6	32 (5)
	W 208		HIM 1004-6	31 (10)
Taxon 45 of Bisgaard	SSI P 876	Taxon 45 of Bisgaard	HIM 1004-6	90 (6)[c]

[a] Standard deviations are given in parentheses.
[b] From Christensen et al. (13).
[c] From Eckert et al. (22) and Stenzel (47).

separated from other taxa by less than 51% DNA reassociation. The limit for defining a species on the basis of DNA reassociation within the family *Pasteurellaceae* has varied between 80 and 85% (2, 10, 38).

Since "*P. multocida* subsp. *tigris*" strain ATCC BAA 600 (8) was found to be a member of taxon 45 and strain SSI P 876, tentatively named "*P. leonis*" (47), also belongs to this taxon, priority should be given to SSI P 876 in future studies on the final classification of this taxon. However, it is of vital importance that the present knowledge be used to preserve additional strains from large-cat bite wounds to allow final classification of organisms classified as taxon 45 of Bisgaard.

The polyamine pattern of SSI P 876 deviated significantly from those of the type strains of the subspecies of *P. multocida* (7). The members *P. multocida*, *P. canis*, *P. dagmatis*, *P. stomatis*, and *Pasteurella* species strain B of 16S rRNA cluster 3B (21) and strain SSI P 876 mentioned above could all be separated from most other members of the family *Pasteurellaceae* by the presence of the polyamine *sym*-nor-spermidine (7), suggesting that strain SSI P 876 is part of 16S rRNA cluster 3B.

Strains HIM 1004-6 and W 208 were included in the study of Schmid et al. (45). Despite their phenotypic variation from *P. multocida*, both strains were found to be closely related to the type strains of the *P. multocida* subspecies by crossed immunoelectrophoresis analysis, in accordance with the results obtained in the present study, including the fact that they belong to capsular type A.

Strains tentatively named taxon 46 seem to represent a new species-like group within the genus *Pasteurella* sensu stricto. These organisms are urease positive and might easily be misidentified as *P. dagmatis* since they are associated with cat-bite lesions in humans. However, this taxon differs from *P. dagmatis* in (−)-D-sorbitol, sucrose, and trehalose fermentation; and laboratories that come across similar organisms are encouraged to keep these isolates or submit them to reference laboratories to enable final studies on the classification of this taxon.

The G+C contents of strains SSI P 876 and HIM 1004-6 were found to be 37.2 and 41.8 mol%, respectively, and the genome masses were found to be 1.8 and 1.7 GDa, respectively (22, 47). These values are within the range of G+C contents of 37.7 to 45.9 mol% and genome masses of 1.4 to 1.9 GDa reported for *Pasteurella* sensu stricto, even though the G+C content of strain SSI P 876 is slightly below this limit.

Surprisingly, all strains of Bisgaard taxon 45 tested positive for the *P. multocida* species test of Miflin and Blackall (35),

and the reasons for this remain to be investigated. The test is based on the 23S rRNA gene sequence as a target for the PCR, and sufficient variation might not be present within this gene for separation of *P. multocida* from taxon 45. Further elaboration of DNA sequence-based tests might contribute to a more accurate means of identification of *P. multocida* and related members of the genus *Pasteurella*.

In conclusion, the present study showed that sucrose-negative variant strains of *P. multocida*-like bacteria, including isolates obtained from large cats, belong to two taxa. One group of strains mainly isolated from cows with pneumonia belonged to *P. multocida*, whereas the other strains mainly isolated from human bite wounds caused by large cats belonged to a new taxon (taxon 45 of Bisgaard). Two other strains isolated from bite wounds caused by leopards formed a new taxon of *Pasteurella* sensu stricto (taxon 46 of Bisgaard) with similarity to *P. dagmatis*. The study showed the limitations of phenotype-based tests as well as those of genotype-based tests, such as 16S rRNA sequence comparison and PCR, for the identification of these bacteria. For the identification of isolates with doubtful identities, the use of a combination of phenotype- and genotype-based tests test is recommended. Only with the collection and deposition of more isolates like these will further characterization in reference laboratories be able to be performed and the taxonomy of these bacteria improved. Further elaboration of DNA sequence-based tests, including PCR, might then contribute to a safer means of identification of *P. multocida* and related members of the genus *Pasteurella*.

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